A high diffusion coefficient for coenzyme Q_{10} might be related to a folded structure

Salvatore Di Bernardo[a], Romana Fato[a], Rita Casadio[b], Piero Fariselli[b], Giorgio Lenaz[a,*]

[a] *Laboratory of Bioenergetics, Department of Biochemistry 'G. Moruzzi', via Irnerio 48, University of Bologna, 40126 Bologna, Italy*
[b] *Laboratory of Biophysics, Department of Biology, via Irnerio 42, University of Bologna, 40126 Bologna, Italy*

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Abstract **We measured the lateral diffusion of different coenzyme Q homologues and analogues in model lipid vesicles using the fluorescence collisional quenching technique with pyrene derivatives and found diffusion coefficients in the range of 10^{-6} cm^2/s. Theoretical diffusion coefficients for these highly hydrophobic components were calculated according to the free volume theory. An important parameter in the free volume theory is the relative dimension between diffusant and solvent: a molecular dynamics computer simulation of the coenzymes yielded their most probable geometries and volumes and revealed surprisingly similar sizes of the short and long homologues, due to a folded structure of the isoprenoid chain in the latter, with a length for coenzyme Q_{10} of 21 Å. Using this information we were able to calculate diffusion coefficients in the range of 10^{-6} cm^2/s, in good agreement with those found experimentally.**

Key words: Coenzyme Q; Molecular dynamics; Phospholipid vesicle; Diffusion; Fluorescence quenching

1. Introduction

Diffusion in biological membranes plays a vital role in a variety of cellular and biochemical processes. Depending on the size of the diffusant with respect to solvent molecules, different theories for the lateral diffusion have been developed and tested. With a diffusant comparable in size to the solvent, the 'free volume theory' of Cohen and Turnbull [1] seems to apply best. According to this theory, a spectrum of free volume sizes is formed by solvent density fluctuations: a diffusing molecule can undergo a diffusive step if a free volume exists adjacent to it of a certain minimum size.

This model can be applied to the diffusion of lipophilic quinones in membrane lipids. The lateral diffusion of quinones in lipid bilayers has received particular attention in relation to their role in electron transfer processes in the mitochondrial respiratory chain; according to the 'random collision model' of the electron transfer proposed by Hackenbrock et al. [2] all components of the mitochondrial respiratory chain are randomly distributed in the plane of the membrane and undergo independent lateral diffusion; the mobility of the smaller components, such as coenzyme Q (CoQ) and cytochrome *c*, is faster than that of the macromolecular complexes and assures electron transfer by random collisions with the latter. Moreover, Hackenbrock et al. [2–4] suggested that CoQ diffusion in the mitochondrial membrane is the rate limiting step in the whole electron transfer process.

A variety of techniques have been employed to measure the lateral diffusion in artificial lipid bilayers and in natural membranes, yielding a broad range of values for the diffusion coefficients D_1. The methods are based either on measuring the frequencies of encounter between probe molecules, or on the generation of a spatial gradient of labeled molecules in the membrane followed by measurement of the rate of probe redistribution. The collision-dependent methods measure lateral diffusion to distances of several nm and include excimer formation [5] or fluorescence quenching [6], whereas the methods based on the redistribution of probe molecules measure diffusion on a μm scale for which the most versatile technique is fluorescence recovery after photobleaching (FRAP) [7]. Hackenbrock et al. [2] and Rajarathnam et al. [8] exploiting the FRAP technique with fluorescent labeled ubiquinone analogues calculated diffusion coefficients in mitochondrial membranes in the range of 10^{-9} cm^2/s. On the other hand, exploiting collisional fluorescence quenching of membrane-partitioned anthroyl-stearate fluorophores by oxidized CoQ homologues, Fato et al. [9] calculated diffusion coefficients $> 10^{-6}$ cm^2/s in both liposomes and mitochondrial membranes, using calculations derived from Lakowicz and Hogen [10] to account for partition and effective membrane concentration of the quencher and the Smoluchowski relation for calculating the diffusion coefficient from the second-order rate (quenching) constant.

Subsequently, using pyrene as a probe and exploiting a modified Stern-Volmer relation for two dimensions and a corresponding bidimensional equation for calculating D_1, Blackwell et al. [11] calculated coefficients of $> 10^{-7}$ cm^2/s for plastoquinone in lipid vesicles. Using the latter relation Lenaz [12] recalculated coefficients of $> 10^{-7}$ cm^2/s on the previous experiments of Fato et al. in lipid vesicles [9].

The aim of this work is to show that a fast diffusion coefficient is consistent with the free volume model and with the finding of surprisingly similar dimensions for the different quinones obtained by molecular dynamics (MD) simulations.

2. Materials and methods

The coenzyme Q homologues were kind gifts from Eisai Co., Tokyo, Japan.

Mixed soybean phospholipids (asolectin) were purchased from Sigma, St. Louis, MO and were purified with the method of Kagawa and Racker [13]. The fluorescent probes pyrene (Pyr), 1-hexadecanoyl-2-(1-pyrenehexanoyl)-*sn*-glycero-3-phosphocholine (Pyr-PC_6) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphocholine (Pyr-PC_{10}), were purchased from Molecular Probes, Junction City, OR and stored as solutions in absolute ethanol at −20°C at a concentration of 1 mM. Phospholipid vesicles were prepared from a stock solution of purified asolectin as described [9]. The final concentrations were 10–30 mg of phospholipids/ml as detected by the phosphorus content [14]. Pyrene was incorporated into the lipid vesicles by addition of the ethanolic solution to an aqueous suspension of vesicles in

*Corresponding author. Fax: (39) (51) 351217.
E-mail: lenaz@biocfarm.unibo.it

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10 mM Tris-Cl^- buffer, pH 7.4 at a probe:phospholipid ratio of 1:400 (mol:mol), and then the mixture was incubated at room temperature for 30 min, while the probes Pyr-PC_6 and Pyr-PC_{10} were cosonicated with the phospholipids. Static fluorescence measurements were carried out on a JASCO FP 777 spectrofluorometer, and time-resolved fluorescence in an IBH single photon counting spectrofluorometer. The excitation wavelengths were 342 nm for Pyr, 346 nm for Pyr-PC_6 and Pyr-PC_{10}. The fluorescence lifetimes were determined by time-resolved fluorescence spectroscopy using the single photon time-correlating technique. The decay curves were analyzed by iterative deconvolution with global analysis procedures [15].

Least squares analysis was carried out using the program CURFIT listed by Bevington [16], which uses the Marquardt algorithm.

Coenzyme Q homologues and analogues were incorporated into the lipid vesicles by addition of different amounts of an ethanolic solution of quinones or by cosonication with the phospholipids.

The fluorescence quenching due to the oxidized ubiquinone homologues and analogues is largely a collisional quenching mechanism as demonstrated by the linearity of the Stern-Volmer plots and by the agreement between steady-state and time-resolved fluorescence quenching experiments.

The diffusion coefficients, for a 3-D diffusion, were calculated from the values of k_m, the bimolecular quenching constant in the membrane phase [9], through the equation of Smoluchowski as modified for fluorescence encounters [10].

By using the above relations, it was possible to calculate only the sum of the diffusion coefficients of the probe plus quencher; we measured the diffusion of the pyrene probes by the collisional excimer technique [5].

The theoretical calculation of the diffusion coefficient for different CoQ homologues followed the free volume theory [1]:

$$D = D^* \exp[-V_0/(V-V_0)]$$

where D^* is the diffusion coefficient for an ellipsoid moving lengthwise in a phospholipid bilayer [17], and the exponential term represents the probability of finding a local free volume of a certain size; V_0 is the approximated molecular volume derived from molecular dynamic simulations and V is the available volume in the lipids at constant room temperature, considering a half thickness of the membrane of ~20 Å and considering the acyl chains of the lipids forming a disk of diameter ~7.5 Å [18].

The stable conformation of the CoQ homologues was studied by energy minimization and MD simulation of initial conformations constructed using standard bond lengths and angles. The starting structure was an all-*trans* configuration of the isoprenoid side chain as determined by NMR experiments [19].

After a first minimization the structure was heated in 0.1 ps by a 0.5 fs MD simulation to 350 K. This temperature was kept as the simulation temperature for 900 ps and then the system was cooled down to room temperature. The system was coupled to a bath to allow a constant simulation temperature. The resulting structure was then energy minimized by a Polak-Ribiere (conjugated-gradient) algorithm yielding the most stable configuration for the quinone.

For the CoQ_{10} homologue an MD simulation at a temperature of 800 K was also performed, furthermore a Monte Carlo conformational search using the force-field MM+ was carried out.

For all the quinone homologues computations were achieved using the force-field MM+ and AMBER (MACROMODEL).

3. Results

Oxidized quinones quench fluorescence of different probes by a collisional mechanism [9] according to the Stern-Volmer relation.

The concentration of quinones used as quenchers was calibrated in the range 1–50 mM in the lipid phase, depending on the homologue or analogue employed, and the dilution of the probe was 1:400 (mol probe:mol lipids). Under these conditions the results obtained in steady-state and time-resolved fluorescence were in good agreement, indicating the collisional nature of the quenching.

The diffusion coefficients for different quinone homologues and analogues, reported in Table 1, are in the order of 10^{-6} cm^2/s.

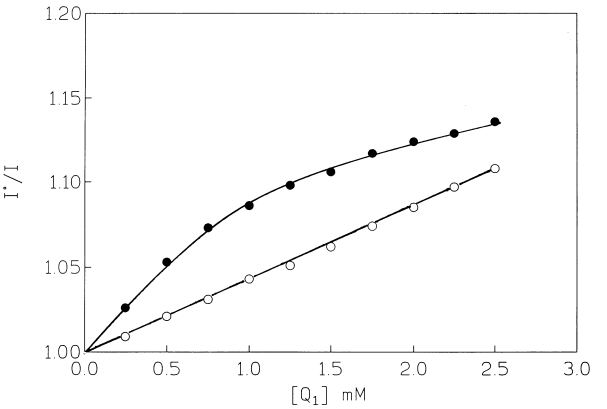

Fig. 1. Quenching of the fluorescence of pyrene (●) and pyrene-PC_{10} (○) by CoQ_1 in liposomes. The two quenching curves were obtained in the same experimental conditions at 0.1 mg PL/ml and the concentration of CoQ_1 in the membrane was obtained from the partition coefficient [9].

In a series of experiments using Pyr as the fluorescent probe for the quenching we found in the Stern-Volmer plots a deviation from linearity with a downward curvature for all quinone homologues and analogues, in both time-resolved and static fluorescence (Fig. 1). On the other hand, in a parallel series of experiments using Pyr-PC_6 and Pyr-PC_{10} as the fluorescent probes, we found linear Stern-Volmer plots without the downward curvature. This behavior may be due to a different localization of the two probes: Pyr-PC_6 and Pyr-PC_{10} are localized deeply in the membrane whereas Pyr is distributed over the whole thickness of the membrane.

The deviation in the Stern-Volmer plots using the Pyr probe and the finding of a unique value for the diffusion coefficient of quinones having widely different isoprenoid chain length opens the question of the localization and of the configuration of the quinone molecules.

For a diffusant comparable in size to the solvent Cohen and Turnbull [1] developed the free volume theory. According to this theory the high mobility and the small differences found using different quinone homologues and analogues suggest a similarity in the size and shape of this diffusing species. Furthermore the saturation effect observed using the Pyr probe

Table 1
Lateral diffusion coefficients of CoQ homologues in lipid vesicles

	D_1 ($\times 10^6$ cm^2/s)		
	Pyr-PC_6	Pyr-PC_{10}	Pyr
CoQ_1	0.6	0.7	1.3
CoQ_2	1.2	1.3	1.2
CoQ_3	1.2	1.4	1.2
CoQ_{10}	1.3	–	–
DB	0.7	–	–

The values were obtained by steady-state fluorescence quenching of the indicated probes, according to [9]. The diffusion coefficients obtained by the Smolouchowski relation (see text) were corrected for the diffusion of the probes, measured by excimer formation [5]. The steady-state fluorescence quenching was closely matched by time-resolved fluorescence quenching. The fluorescence lifetimes were 108 ± 2 ns for Pyr-PC_6, 112 ± 2 ns for Pyr-PC_{10} and 134 ± 3 ns for Pyr. DB, decyl-ubiquinone. The data are means of at least 3–5 determinations agreeing within 5%.

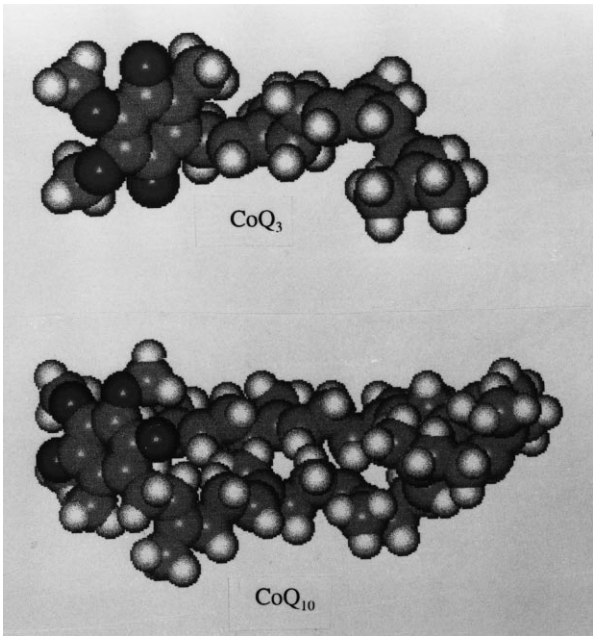

Fig. 2. Structure of the homologues CoQ_3 and CoQ_{10} predicted by MD simulation.

indicates that the diffusion takes place in the very fluid central region of the bilayer.

We carried out computer simulations to determine a possible structure for different CoQ homologues. In Fig. 2 we show the possible configuration for CoQ_{10} and CoQ_3 as obtained by MD simulations and energy minimization of a starting structure constructed using standard bond lengths and angles. Similar structures were obtained also with a Monte Carlo conformational search and small differences were also found for the structures obtained by MD simulation using the two force-fields MM+ and AMBER. It was impossible to obtain a unique global minimum for the folded structure of CoQ_{10} suggesting that the potential configuration surface of the molecule is constituted of multiple local minima with low energy differences between them. In any case the folded structure was always achieved starting from different initial configurations. Furthermore a significant energy difference was obtained between the folded and extended structures of CoQ_{10}, the former having lower energy (Table 2). In Table 2 we also present the volumes calculated from the molecular models and the diffusion coefficients calculated from those volumes according to the free volume theory.

As can be seen from Table 2, the D_1 values are in good agreement with those found experimentally, whereas considering a fully extended CoQ_{10} molecule the theoretically determined diffusion coefficient would drop by one order of magnitude. The calculations and the experimental data hence suggest a highly folded CoQ_{10} molecule may be the most probable one, a consideration also supported by the finding of a significant energy difference between the folded and extended configurations of the CoQ_{10} molecule, and that the molecule is localized in the midplane of the double layer.

4. Discussion

The differences in the D_1 coefficients observed using different techniques are the result of the complex interrelation between different kinds of diffusion (short-range and long-range diffusion), the dimensions and the localization of the diffusing quinones.

The monomeric quinones that enter the membranes of the vesicles used as the model system seem to move faster than the lipid molecules that solubilize them (as mimicked by the motion of the fluorescent phospholipids, PC_6 and PC_{10}, revealed with the excimer technique).

This apparent paradox could be explained considering that biological membranes are complex anisotropic fluids in which molecules display a large variety of motional dynamics. The lipid hydrocarbon domain may be represented as a newtonian fluid. In this domain the interaction energy among the hydrocarbon chains is relatively low [20]. Accordingly, the fluidity in this domain is relatively high. In contrast, at the hydrocarbon-water interface and at the hydrophilic boundary the dominant interactions are ionic or hydrogen bonds and the fluidity in these regions will be determined primarily by the strong intermolecular forces.

The fast motions like *trans-gauche* isomerization of hydrocarbon phospholipid chains account for the formation of 'voids' in the midplane of the membrane with a frequency compatible with the jump frequencies of the quinones diffusing in the membrane. Actually in this central region of the membrane the lateral displacement of the lipids is very fast, corresponding to a lateral diffusion coefficient of 10^{-6} cm^2/s, as calculated by computer simulations [20,21].

These considerations suggest a deep localization of the quinones in the membrane core, supported by the finding that, in experiments that use Pyr as the fluorescent probe, the Stern-Volmer plots present a downward curvature. The high hydrophobicity of the quinones localizes them in the center of the membrane making more superficially localized probe molecules to be less quenched. This hypothesis is supported by the observation of a stronger deviation from linearity in the case of quinones with a longer hydrophobic tail, and demonstrates the presence of inaccessible fluorophores localized near the surface of the membrane; thus since Pyr is randomly distributed over the whole thickness of the bilayer and since the effect is more evident for those quinones having longer hydrophobic tails, it is correct to localize those inaccessible fluorophores near the surface of the membrane.

If we regard 1×10^{-6} cm^2/s as the correct value for the D_1 of the quinones in the membrane, the probe would scan a

Table 2
Diffusion coefficients calculated according to the free volume equation

	CoQ_1	CoQ_2	CoQ_3	CoQ_{10} folded	CoQ_{10} extended
V_0 (Å^3)	109.30[a]	150.72[a]	213.52[a]	351.70[a]	628.00
E[b]	27.2	30.5	37.9	56.1	61.3
D ($\times10^{-6}$ cm^2/s)	3.30	2.20	1.40	1.43	0.34

[a]From structures obtained by MD simulations.
[b]Potential energy expressed in kcal/mol.

distance of 6.3 nm during its lifetime of 100 ns, which is more than twice the dimension of the diffusing species.

The structure of an extended CoQ_{10} proposed in preceding works [22] is in disagreement with our finding of a high, unique diffusion constant for all quinone homologues and analogues used in the quenching experiments. It is difficult to fit the fully extended CoQ_{10} molecule, with its 50 Å length, in the bilayer with an orientation perpendicular to the plane of the membrane. This orientation allows the lateral movement of the molecule, but the diffusion coefficient calculated would be inconsistent with those coefficients experimentally found for the probes and lipid diffusion. In addition the necessary flip-flop of the quinone ring from one to the other face of the membrane would have a high activation energy, in contrast to NMR experiments showing a fast flip-flop rate for the CoQ_{10} headgroup [23].

The necessity of a partly folded configuration for the CoQ_{10} was suggested in previous work by Lenaz et al. [12,24]; this model would account for the transfer of redox equivalents from one to the other side of the membrane, but the dimensions are still too large to fit with a free volume theory of the diffusion.

The results obtained with our experiments and the structure found for the quinones by computer simulations with the surprisingly similar dimensions between different CoQ homologues can reconcile the different aspects of the localization and motion of the lipophilic component of the mitochondrial respiratory chain.

Furthermore, the maximal dimension of 21 Å found for CoQ_{10} gives us the certainty that we are really measuring a short-range diffusion using Pyr, and derivatives, as fluorescent probes and not only an oscillation near the equilibrium position as it would be if we consider a 50 Å dimension of an extended CoQ_{10} molecule.

Since the mean distance between the redox complexes in the inner mitochondrial membrane is in the order of 30 nm [12], we consider a diffusion over this distance significant for the electron transfer process in the mitochondrial respiratory chain and hence a technique measuring short-range diffusion, such as the pyrene fluorescence quenching technique, would be the most appropriate one.

The implications of a folded structure of CoQ_{10} for electron transfer are manifold: protein binding of ubiquinone during electron transfer may require unfolding, contributing to a high activation energy [25] and low collision efficiency [26] of electron transfer in CoQ-reactive enzymes.

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